AD_____

GRANT NUMBER DAMD17-94-J-4065

TITLE: The Roles of TGF-Beta and TGF-Beta Signaling Receptors in Breast Carcinogenesis

PRINCIPAL INVESTIGATOR: Xiao-Fan Wang, Ph.D.

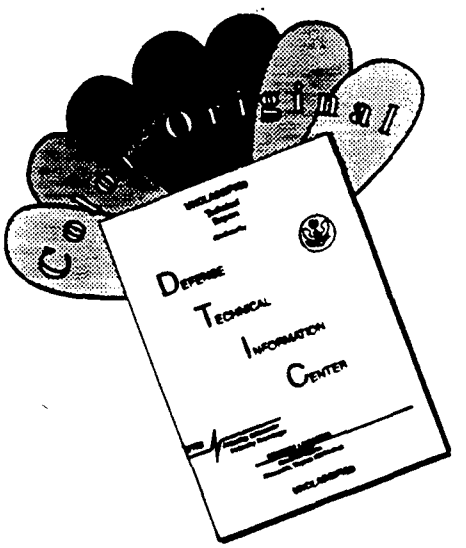
CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27708

REPORT DATE: July 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF COLOR PAGES WHICH DO NOT REPRODUCE LEGIBLY ON BLACK AND WHITE MICROFICHE.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Annual (1 Jul 95 - 30 Jun 96)

4. TITLE AND SUBTITLE
The Roles of TGF-Beta and TGF-Beta Signaling Receptors in Breast Carcinogenesis

5. FUNDING NUMBERS
DAMD17-94-J-4065

6. AUTHOR(S)
Xiao-Fan Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27708

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The overall goal of this research project is to explore the roles of TGF-ß and components of its signaling pathways in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. We have identified and isolated two cDNAs encoding members of the Dwarfins family and studied the TGF-ß induced phosphorylation of these two molecules in a normal mammary epithelial cell line. Further studies are underway to address the functional significance of the induced phosphorylation in the mediation of the TGF-ß growth inhibitory signal. We have also investigated the importance of a paracrine loop involving the interactions between mammary epithelial and fibroblast cells. We specifically studied the regulation of IGFBP3 expression in the presence of TGF-ß. The results indicated that, in addition to its direct effect on the cell cycle progression, TGF-ß may have a broader role in affecting the epithelial cell proliferation through its induction of IGFBP3 in fibroblast, consequently blocking the action of mitogenic factors, such as IGF, in breast tissues. Results from further analysis in this direction will not only significantly contribute to an understanding of the molecular events leading to breast carcinogenesis, but also aid in the development of new therapeutics for breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, TGF-beta, IGF, IGFBP, Cell Cycle, Signal Transduct.	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

__X__ Where copyrighted material is quoted, permission has been obtained to use such material.

__X__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__X__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__X__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

__X__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__X__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__X__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

7/26/96

PI - Signature Date

Table of Contents

A. INTRODUCTION

Breast cancer is the most common cancer in women in the United States. Endocrine therapy has proven to be beneficial in approximately one-third of breast cancer patients. However, the tumors inevitably progress to a state of hormone insensitivity and no longer respond to conventional endocrine therapies. Therefore, it is necessary to identify other molecular markers to monitor the pathological process of the disease in order to better evaluate patient prognosis and to elucidate molecular mechanism of breast cancer initiation, progression and metastasis in order to develop new reagents for subsequent treatments. The proposed study of TGF-ß growth inhibitory signaling pathways in breast carcinogenesis will significantly benefit both of these purposes.

The overall goal of this research project has been to explore the roles of TGF-ß and components of its signaling pathways in the initiation, progression and metastasis of breast adenocarcinomas through an investigation of the disregulation of TGF-ß signal transduction. While breast cancers almost exclusively originate in epithelial cells, they cannot develop significantly without manipulating and recruiting the activities of surrounding vasculature, matrix proteins, and stromal components. Of particular importance to the genesis and progression of breast cancers are the stromal/epithelial interactions, as these interactions have been found to be important to both neoplastic and normal development of mammary tissue (1). Normal growth of breast epithelium is tightly regulated by a complex system of negative and positive autocrine and paracrine controls. Involved in this control are the growth promoting factors such as the hormones, estrogen and progesterone, the IGFs, and the growth inhibiting factors such as the TGF-ßs. Although it is known that tissue growth homeostasis is maintained by communication between the stromal and epithelial cells, the molecular details of these interactions are poorly understood. It is clear, however, that aberration of this homeostasis can lead to the development of a neoplastic state.

TGF-ßs are a group of multifunctional polypeptide hormones which play important roles in many normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix formation, cell adhesion and migration (2,3). Additionally, TGF-ß acts to inhibit the proliferation of a variety of cell types, including breast epithelium, by playing a pivotal role in maintaining negative autocrine and paracrine loops (4). Therefore, lesions to TGF-ß signaling pathways that disrupt the negative growth regulation of breast epithelia may contribute to mammary carcinogenesis and represent an obligatory step in neoplastic progression of breast epithelia. Indeed, loss of TGF-ß responsiveness of MCF-7 breast cancer cells has been shown to correlate with a loss of expression in these cells of the TGF-ß signaling receptors (5). Loss of such autocrine control by TGF-ß represents an opportunity for malignant epithelia to increase proliferation in response to other positive growth factors, like IGF, and hormones, like estrogen.

Elucidation of signaling mechanism by the TGF-ß receptor complex and the discovery of a molecular link between the TGF-ß signal and the cell cycle control machinery have significantly advanced our knowledge of the molecular nature of the TGF-ß growth-inhibitory signaling pathway. However, the intracellular signaling cascade through which TGF-ß signals remains largely unknown. A number of molecules which interact with the receptor kinases and serve as in vitro substrates have been isolated through the two-hybrid system in yeast (6-9). The functional significance of those interactions, however, is completely unclear since the phosphorylation events have not been observed to be regulated by TGF-ß in vivo and there is no indication of how these interacting molecules could potentially be involved in TGF-ß signaling. In another study, a kinase, termed TAK which is similar to the MEK kinase in the MAP kinase signaling pathway, was shown to affect TGF-ß signaling when transiently overexpressed (10). The precise mechanism of TAK's involvement in TGF-ß signaling remains to be elucidated.

Genetic analyses in *Drosophila* and in *C. elegans* have led to the isolation of TGF-ß-like pathway components, including ligands and receptors which are homologous to those identified in vertebrates (11). Recently, a group of molecules termed Dwarfins have been identified in these invertebrates (MAD in *Drosophila*, SMA-2, SMA-3, and SMA-4 in *C. elegans*) which potentially serve as downstream effectors in the TGF-ß-like signaling pathway, since mutations in these genes cause the same mutant phenotype as the mutant receptors of the TGF-ß-like ligands (12,13). Evidence which further supports the notion that the Dwarfins may be involved in growth-inhibitory pathways, such as that of TGF-ß, came from genetic analyses performed in humans. A candidate tumor suppressor gene, DPC4, was found to be mutated in more than 50% of human pancreatic cancers (14). The molecular structure of the DPC4 gene product suggests that it belongs to the Dwarfin family with more than 60% sequence homology to the Mad and the Sma genes. In addition, DPC4 contains two highly conserved domains, termed DH1 and DH2, which are shared by all known Dwarfins from invertebrate to mammals (13).

While genetic analyses have established that the Dwarfins function as potential downstream effectors of the signaling pathways for ligands of the TGF-ß superfamily, the biochemical nature of the signaling mechanism by which the Dwarfins transmit the TGF-ß signal remains poorly understood. Furthermore, the potential involvement of Dwarfins in the control of normal cell proliferation as well as the pathological process of breast carcinogenesis in mammary epithelium needs to be investigated. To address this question, we have recently isolated the full length cDNA clones for two murine Dwarfins, A & C, and examined their functional involvement in the mediation of TGF-ß growth-inhibitory signal in a murine mammary epithelial cell line NMuMg. The results from these studies are summarized in the next section (a preprint describing the findings is enclosed in the Appendix).

The insulin-like growth factors, IGF-I and IGF-II, are potent mitogens for a wide variety of cells including breast tumor epithelium. The importance of the IGFs in breast cancer has been supported by studies demonstrating that treatment of a breast cancer epithelial cell line, MCF-7, with monoclonal antibodies against the type I IGF receptor, blocks the mitogenic effects of both IGF-I and IGF-II (1). Other studies have shown that antibody treatment against the type I IGF receptor can actually inhibit the formation of mammary tumors grown in nude mice (15). The source of IGF production in mammary tissue is stromal fibroblasts, once again emphasizing the importance of paracrine signaling between stromal and epithelial cells in the regulation of breast cancer epithelial cell growth.

The functions of IGF family is regulated by a series of binding proteins, termed IGFBPs, which have been shown to circulate in extracellular fluids and modulate the proliferative and mitogenic effects of IGFs on cells (16). By binding the IGFs with high affinity, the IGFBPs play pivotal roles in regulating the availability and bioactivity of IGFs. It has been further demonstrated that IGFBPs can modulate cellular function in the absence of IGFs, presumably by specific IGFBP cell-surface receptors (17). IGFBP-3 is one of six IGFBPs identified to date (18). Although structurally related, these IGFBPs differ in size, biochemical properties, IGF binding preference, tissue-specificity, hormonal regulation, and presumably, physiological function. IGFBP-3 is expressed in most adult tissues, including mammary tissue, and represents the major circulating IGFBP in adults (19). Consequently, IGFBP-3 present in the cellular microenvironment has the potential to directly alter local cell response to IGF-I and IGF-II. The expression of IGFBP-3 has been shown to be induced in a variety of cell types, including fibroblasts and breast cancer cells, by TGF-ß, anti-estrogens, and retinoic acid (20-22). Thus, induction of IGFBP-3 by TGF-ß in stromal fibroblasts to modulate the IGF mitogenic activity in stimulating the proliferation of breast epithelial cells may represents a crucial paracrine effect of TGF-ß. To this end, we have initiated studies on this topic in the last year and some preliminary results generated are summarized in the next section.

B. PROGRESS REPORT

In the last twelve months, our work has been focused on two main areas: examination of the functions of one component of the TGF-ß signaling pathway, Dwarfins, in the mediation of TGF-ß growth inhibitory signal in mammary epithelial cells; and evaluation of a hypothesis that the induction of IGFBP-3 by TGF-ß in stromal fibroblasts is a mechanism by which TGF-ß regulates the growth of breast epithelial cells. These studies were proposed as Specific Aims 4 & 5 in the original proposal (corresponding to Tasks V & VI in the Statement of Work) and the progress in these areas are reported below.

To investigate the potential role of the dwarfins in TGF-ß or TGF-ß superfamily signaling pathways, we isolated two murine dwarfins, Dwf-A and Dwf-C by low stringency hybridization. The amino acid sequences of Dwf-A and Dwf-C are 92% homologous and 87% identical. Dwf-A and Dwf-C are about 75% homologous to the *Drosophila* dwarfin, MAD (12), and the *C. elegans* dwarfin, SMA-2 (13). Dwf-A and Dwf-C are only distantly related to the only other known mammalian dwarfin, DPC4 (60% homology; 40% identity), and thus likely represent a distinct family of mammalian dwarfins. Dwf-A and Dwf-C contain the characteristic dwarfin homology domain 1 (DH1) and dwarfin homology domain 2 (DH2) motifs separated by a proline-rich linker region. A distinction between the mammalian dwarfins and SMA-2 is two serine/threonine rich inserts in the linker region.

Genetic evidence suggests that a single*C. elegans* TGF-ß-like pathway requires three functionally non-redundant, yet highly related dwarfins, SMA-2, SMA-3 and SMA-4 (13). Because Dwf-A and Dwf-C are members of the same class of dwarfins, it was important to determine if they are tissue-specific or differentially expressed. Northern analysis showed that both genes are ubiquitously expressed (data not shown). The mRNA message of Dwf-A is ~3.5 kb, while Dwf-C has two messages of ~8 kb and ~3 kb. This suggests that multiple dwarfins may be required to propagate TGF-ß or TGF-ß superfamily signals in mammals as well as *C. elegans*.

We have studied the regulation of endogenous Dwf-A and Dwf-C in two cell lines which are growth inhibited by TGF-ß, NMuMg (normal murine mammary epithelial line) and L6 (rat myoblast line). Polyclonal antibodies were produced which preferentially recognize Dwf-A or Dwf-C (data not shown). We used these antibodies to determine if the levels or phosphorylation state of endogenous dwarfins were modulated by TGF-ß. The expression levels of Dwf-A and Dwf-C were unchanged at any time within 24 hours following TGF-ß treatment (data not shown). However, TGF-ß treatment of NMuMg cells results in rapid, but transient phosphorylation of both Dwf-A and Dwf-C (Fig. 2A, Appendix). Immunoprecipitation with the Dwf-A antibody showed a TGF-ß induced phosphorylation of two specific proteins, Dwf-A and a cross-reacting protein of ~54 kDa. The Dwf-C antibody revealed an induced phosphorylation of Dwf-C and a ~54 kDa cross-reacting protein. Phosphorylation of Dwf-A and Dwf-C was induced 2-fold within 15 min and peaked at 3-fold by 30 min (Fig. 2A, Appendix). By 4 hours the phosphorylation state of all three proteins had returned to nearly basal levels. Quantitation of the cross-reacting 54 kDa band from αDwf-A immunoprecipitates showed a 2.5-fold activation within 30 min, while the 54 kDa band from αDwf-C immunoprecipitates showed a 5-fold activation within 30 min. The difference in the extent of phosphorylation of the 54 kDa protein in the two immunoprecipitates is due to the lack of basal phosphorylation in the absence of TGF-ß treatment in the αDwf-C immunoprecipitate (Fig. 2A, Appendix). The induced phosphorylation of Dwf-A and Dwf-C was primarily on serine residues (~90%) with some threonine phosphorylation (~10%) as determined by phosphoamino acid analysis (data not shown).

Immunoprecipitation of Dwf-A and Dwf-C from L6 cells gave nearly identical results as those from NMuMg cells (data not shown). Dwf-A, Dwf-C and the 54-kDa protein were phosphorylated to a similar extent and with similar kinetics from both cell lines. Therefore, dwarfin phosphorylation is a rapid response to TGF-ß in two responsive cell lines.

To ensure that TGF-ß was capable of inducing phosphorylation of the dwarfins at more physiological concentrations we determined the dose dependence of dwarfin phosphorylation in NMuMg cells. TGF-ß at 10 pM resulted in a 2.5-fold increase in Dwf-A phosphorylation in 1 hour with a peak of 3.5-fold at 100 pM (data not shown). Phosphorylation of Dwf-C was increased 3.5-fold by 10 pM TGF-ß in 1 hour and peaked at 4.5-fold at 100 pM (data not shown). The 54 kDa protein in both Dwf-A and Dwf-C immunoprecipitates was also inducibly phosphorylated by 10 pM TGF-ß and peaked at 100 pM. Thus, TGF-ß is capable of modulating the phosphorylation state of endogenous dwarfins at concentrations which are physiologically relevant.

TGF-ß-like ligands from *Drosophila* and *C. elegans* are most closely related to mammalian bone morphogenetic proteins (BMPs) (11). BMPs have been shown to elicit multiple effects on many different cell types including inhibition of cellular proliferation (11). BMP-2 has been shown to initiate its signaling cascade by binding a heteromeric complex of transmembrane serine/threonine kinase receptors at the cell surface (23). This mechanism is analogous to the TGF-ß receptor system (24). However, the similarity between the cytoplasmic pathways that lead to the biological effects of TGF-ß and BMP-2 is unknown. The NMuMg cell line is potently inhibited by BMP-2 (unpublished results) which afforded us the opportunity to determine if dwarfin phosphorylation is induced by BMP-2. Phosphorylation of Dwf-A and Dwf-C is induced 1.6-fold by 100 ng/ml BMP-2 within 15 min and peaks at 2.5-fold in 1 hour (Fig. 4A and B, Appendix). Interestingly, the 54 kDa protein which is inducibly phosphorylated in response to TGF-ß is not phosphorylated in BMP-2 treated NMuMg cells (Fig. 4A, Appendix). This is the first indication that TGF-ß superfamily members may utilize overlapping yet distinct signaling mechanisms to achieve the same phenotypic effect. Efforts are underway to clone and identify the 54 kDa protein which, based on its cross-reactivity, most likely represents another murine dwarfin. The differential response of this protein to TGF-ß and BMP-2 will facilitate elucidation of the differences in the intracellular pathways utilized by TGF-ß and BMP-2.

To address the functional role of the dwarfins in TGF-ß signaling we cloned Dwf-A and Dwf-C into mammalian expression vectors. Attempts to establish stable cell lines constitutively overexpressing either Dwf-A in L6 cells or Dwf-C in NMuMg cells were unsuccessful. This result was not unexpected since the dwarfins are suspected to have growth suppressive effects based on the tumor suppressor activity of DPC4 (14). Consequently, we used a modified transient growth assay (25) to assess the ability of Dwf-A or Dwf-C to cause a growth arrest when transiently transfected into L6 cells. Overexpression of Dwf-A or Dwf-C causes 30-40% growth inhibition compared to 10% for control transfectants (Fig. 5, Appendix). Therefore, Dwf-A and Dwf-C, like DPC4, exhibit growth inhibitory properties, a phenotypic hallmark of TGF-ß.

While the dwarfins do not possess any known catalytic motifs, their DH1 and DH2 domains are reminiscent of SH2 and SH3 domains which, in a variety of signaling pathways, modulate protein/protein interactions based on tyrosine phosphorylation and proline-rich sequences, respectively (26). TGF-ß and BMP-2 induced phosphorylation of the dwarfins may regulate protein/protein interactions in an analogous fashion for TGF-ß superfamily signaling cascades.

As described in the last year's Annual Report, we have continued studies on the examination of specific TGFβ gene responses and growth responses, and the modulation of these

effects by the steroid hormones in breast cell lines (outlined in Specific Aim 3). We have established a breast cell culture system to examine the interactions between the negative growth promoting pathway induced by TGF-ß and the positive signaling pathway(s) of the steroid hormones. We have maintained three separate MCF7 breast cancer cell sublines for our study of TGFβ growth effects. MCF7 cells are ideal for our studies as they are still responsive to hormones and cytokines, thus representing an early stage of breast cancer. As breast cancers progress, the cells involved typically become resistant to both positive and negative growth factors which makes these later stage cancers immune to standard therapeutic methods. However, in mammary epithelium that is still responsive to growth factors, TGF-ß acts primarily as an inhibitor of cell proliferation. We have identified several MCF7 breast cancer cell lines that show a 90% growth inhibition after TGF-ß addition and a 10-fold proliferation increase after addition of steroid hormones (Figure 1). We have verified that these growth effects are due solely to TGF-ß and estrogen by using the breast cancer cell line, SKBR3, as a negative control. The SKBR3 line represents a late stage cancer since it is known to have lost responsiveness to the signalling pathways of hormones and cytokines. Our cell culture system has revealed a point of interaction between the two opposing signals. When MCF7 cells are exposed to both TGF-ß and estrogen, the growth of the treated cells is intermediate between the two effects alone (Figure 1). The cells are neither fully growth inhibited as if by TGF-ß alone nor are they fully responsive to the growth-promoting signal from estrogen Thus, the presence of estrogen blocks the TGF-ß-growth inhibition, yet TGF-ß blocks the full effect of estrogen-induced proliferation, resulting in a net 5-fold proliferation of the cells (Figure 1).

To determine the mechanism by which these two growth regulators interact, we have initially focused our attention on the cell cycle regulators (progress in this part of research was reported in the last year's Annual Report). In other cell systems, the down regulation of the G1 cyclin dependent kinases (specifically cdk2 and cdk4) and their cyclin partners (especially cyclin E) contribute to the growth inhibitory effects of TGF-ß. The G1 cyclins and cyclin dependent kinases (cdk's) play an essential role in the progression of a cell through the G1 phase of the cell cycle. Regulation does not occur solely by alterations in the cyclin/cdk expression levels. In recent years, treatment of different types of cells with growth-inhibiting substances such as TGF-ß cause the increased accumulation of factors called cyclin dependent kinase inhibitors (CkI's) which inhibit cyclin/cdk activity. TGF-ß has been shown to suppress the growth of several different cell types by inducing the expression of various cyclin/cdk inhibitors including, p15 (27), p21 (28), and p27 (29,30).

In our laboratory, we have demonstrated that TGF-ß induces the expression of the CkI's, p15 and p21, in human keratinocyte cells (HaCaT cells, 28,31). Induction of these proteins is correlated with TGF-ß-induced growth inhibition in the HaCat cell line. To determine whether or not TGF-ß induces the expression of one or more of the CkIs in the MCF7 cells, we isolated cytoplasmic mRNA and protein lysates from cells treated with TGF-ß and assayed for an increase in p15, p21, and p27 mRNA and protein levels. We have found that p15, not p21, appears to be a major component of TGF-ß-mediated growth inhibition in MCF7 cells. In as little as 7 hours after TGF-ß addition, the levels of p15 mRNA increase approximately 10-fold (Figure 2). As to be expected, the presence of estrogen does not increase p15 mRNA levels. However, when MCF7 cells are exposed to both estrogen and TGF-ß, the positive growth signals initiated by estrogen block TGF-ß's induction of p15 mRNA (Figure 2). This block in p15 mRNA induction correlates with the growth effects observed in the MCF7 cells after TGF-ß and estrogen addition. Thus, p15 represents an intersection of both pathways through which the growth of the breast epithelial cells may be manipulated. Interestingly, we have also found that other mitogenic substances including the insulin-like growth factors (IGF1 and IGF2) block p15 mRNA induction by TGF-ß in a similar manner, supporting their roles as common mitogenic growth factors (Figure 2). We are currently investigating whether estrogen and the IGFs affect TGF-ß-induced p15 expression through a direct or indirect mechanism. Continued examination

of this observation may lead to novel growth regulators that are affected during cancer progression.

Our work on this project has progressed so rapidly that we have begun to investigate an additional aspect of TGF-ß-induced growth inhibition in the breast cancer epithelial cells. The induction of p15 in the MCF7 cells is an example of TGF-ß's direct effect on the cell cycle machinery in order to slow the growth of the breast cancer epithelial cells. In addition to these effects, TGF-ß appears to have a broader effect on breast cell populations through paracrine actions. While breast cancers almost exclusively originate in epithelial cells such as the MCF7 cells, they cannot develop significantly without the surrounding stromal components. The interactions between the stromal fibroblasts and the mammary epithelial are particularly important in both neoplastic and normal development of mammary tissue (for review, see 32). Involved in these stromal/epithelial interactions are the growth promoting factors such as the steroid hormones and the insulin-like growth factors (IGFs), and the growth inhibiting factors such as the TGF-ß's. We have expanded our studies of TGFβ-induced growth inhibition in the breast cancer by examining TGF-ß's role in the stromal/epithelial paracrine loop. Specifically, we have begun to focus on the roles of TGF-ß and IGF1 in the progression of breast cancer.

The IGFs have been extensively characterized and have been shown by several groups to be potent mitogens for breast epithelial cells. The importance of the IGFs in breast cancer has been supported by studies in MCF7 cells demonstrating that antibodies against the type I IGF receptor (recognized by both IGFs) block the mitogenic effects of both IGFs (32). Furthermore, type I IGF receptor antibodies reduce the formation of mammary tumors grown in nude mice (15). The stromal fibroblasts are the main source for IGF production in mammary tissue, emphasizing their importance in the development of breast tumors.

TGF-ß has been found to induce the expression of insulin-like growth factor binding protein 3 (IGFBP3) in a variety of cell types, including fibroblasts and breast cancer cells (20-22). IGFBP3 belongs to a family of secreted proteins that modulate and suppress the growth-stimulatory effects of IGF1 and IGF2 (for review, see 16). By binding to the IGFs with high affinity, the IGFBPs play pivotal roles in regulating the availability and bioactivity of the IGFs. IGFBP3 is expressed in most adult tissues, including mammary tissue, and it represents the major circulating IGFBP in adults (16,19). We intend to test the hypothesis that a stromal/epithelial paracrine loop exists between TGF-ß, IGF1, and IGFBP3.

We have established a collaboration with Dr. John Fowlkes from Duke Medical Center's Department of Pediatric Endocrinology to test this potential paracrine role of TGFβ in breast cancer cells. Dr. Fowlkes has given us a fetal lung fibroblast cell line, MRC9, that secretes insulin-like binding protein 3 (IGFBP3) in the presence of TGF-ß. MRC9 cells are not growth inhibited by TGF-ß, yet TGF-ß induces a 20-fold increase in both IGFBP3 mRNA and protein in these cells (Figures 3 and 4). We are currently using these cells to represent stromal fibroblasts in our cell culture system. Using the fibroblasts together with the epithelial cells, we intend to demonstrate that TGF-ß suppresses the growth of the breast epithelial cells in two ways: 1. directly, by affecting the cell cycle machinery (as described above); and 2. indirectly, through a paracrine pathway, by inducing the fibroblasts to secrete IGFBP3 which, when released into the environment surrounding breast epithelial cells, block the IGF-mediated stimulation of the epithelial cells. In addition, we are interested in defining the mechanism(s) involved in the TGFβ-induced expression of IGFBP3 in the MRC9 cells and in other fibroblasts. We have already begun to identify the potential TGF-ß regulatory elements within the IGFBP3 promoter that control its tissue specific induction in the fibroblasts (33,34). These additional project goals will not only supplement our original investigation into the mechanisms of TGF-ß growth inhibition of breast epithelial cells, but they will provide a novel cell culture model of the stromal fibroblast/epithelial interactions observed in clinical breast cancer cases.

C. CONCLUSIONS

Significant progress has been made in advancing the goal described in the Specific Aim 4 of the original proposal. Specifically, our studies have demonstrated that at least two members of the dwarfin family, Dwf-A and Dwf-C, are rapidly phosphorylated in response to TGF-ß and BMP-2. Furthermore, these proteins cause growth inhibition when overexpressed in a mammalian cell line. Together with the genetic evidence from *Drosophila* and *C. elegans* implicating Dwarfin family members in TGF-ß-like signaling pathways, our results establish the dwarfins as effectors of growth inhibitory signals initiated by TGF-ß and BMP-2 in mammals. Our results further suggest that overlapping, yet distinct dwarfins, may be involved in TGF-ß superfamily signaling cascades. Future studies may reveal if members of the Dwarfins family play significant role in the pathological process of breast carcinogenesis, resembling the role of DCP4, a member of the Dwarfins family, in pancreatic carcinogenesis.

As described in the second part of the report, cell growth is dictated by a delicate balance between positive and negative extracellular proliferative signals. Loss of this balance is a key factor leading to cancer. The goal of this part of research (Specific Aim 5 in the original proposal) aims to determine the role of the antagonistic relationship between two opposing growth signals, IGF and TGF-ß, in mammary tumorigenesis. These two factors represent opposing forces which contribute to the balance of proliferation and growth inhibition of normal mammary growth and development of normal mammary growth and development. As discussed above, TGF-ß acts to directly affect cell cycle progression and cause a G1 cell cycle arrest by activating several cyclin dependent kinase inhibitors, including p15, p21, and p27. In addition to these effects, TGF-ß has a broader effect on cell populations through paracrine actions. It has been recently shown that TGF-ß can induce the expression of IGFBP-3, a protein which inhibits the action of the mitogenic IGFs. We have started to test this hypothesis by establishing a model system in which the effects of TGF-ß through IGFBP-3 can be studied. We intend to subsequently define the molecular mechanisms by which TGF-ß induces IGFBP-3 by identifying TGF-ß regulatory elements in the IGFBP-3 promoter and the corresponding cellular components acting at these promoter elements.

D. APPENDIX

Yingling, J. M., Das, P., Savage, C., Zhang, M., Padgett, R. W., Wang, X.-F. (1996) Mammalian Dwarfins are phosphorylated in response to TGF-ß and are implicated in control of cell growth. Proc. Natl. Acad. Sci. USA, in press.

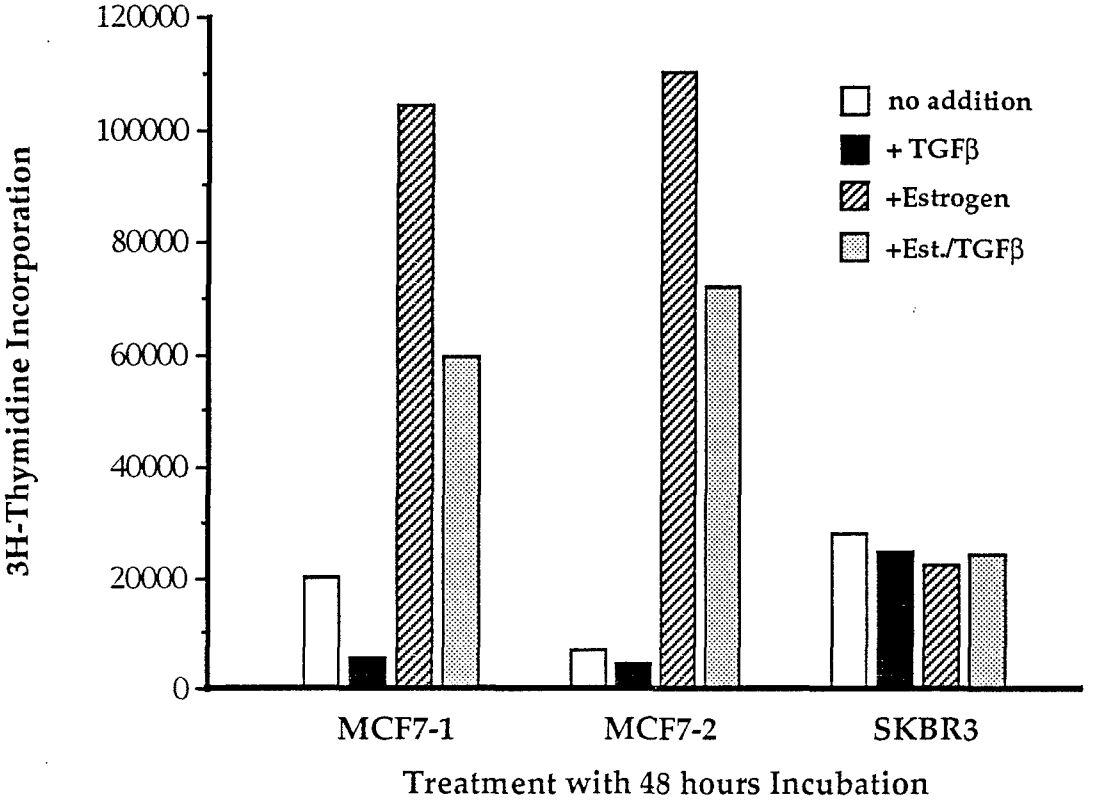


Figure 1. Effect of TGFβ and estrogen on MCF7 and SKBR3 cell proliferation. Cells were incubated for 48h with TGFβ, estrogen, TGFβ and estrogen or with no treatment (see legend). After 48h, 4 μC of 3H-thymidine was added to the media, and the cells were incubated for an additional 3h. The cells were then lysed and assayed for 3H-thymidine incorporation. Two MCF7 cell clones were used for the assay, MCF7-1 and MCF7-2.

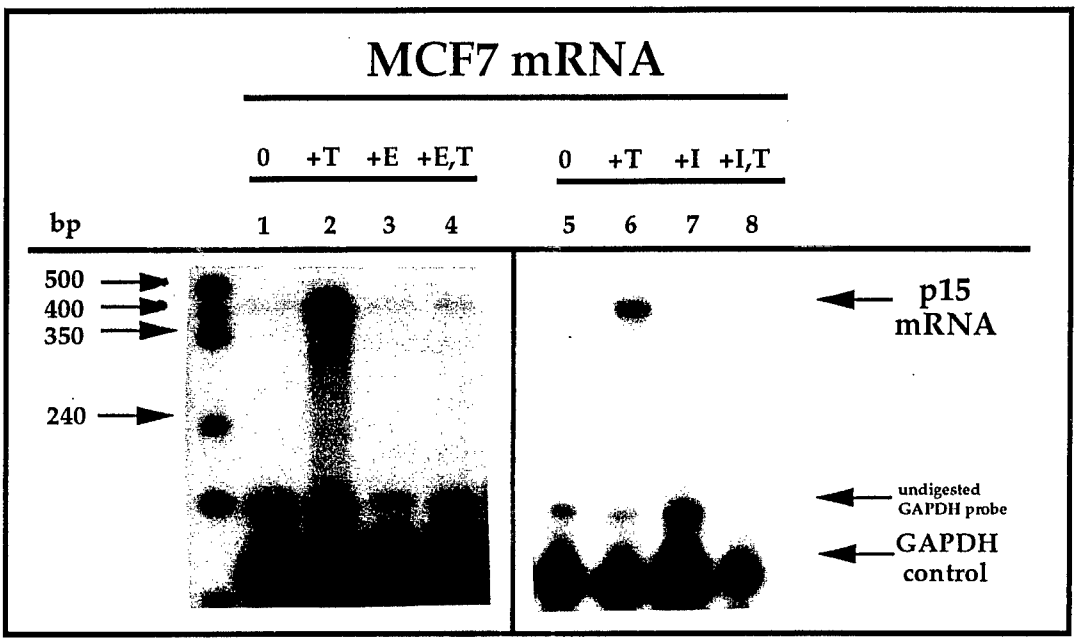


Figure 2. Expression of p15 mRNA in MCF7 cells after simultaneous incubation with TGFβ and the mitogens, estrogen and IGF1. MCF7 cells were incubated for 26h (lanes 1 - 4) and 17h (lanes 5 - 8) with and without TGFβ (lanes 1, 2, 5, and 6), estrogen or IGF1 (lanes 3 and 7), and a combination of TGFβ and estrogen (lane 4) or IGF1 (lane 8). After incubation, cytoplasmic RNA was isolated and hybridized with both a p15 and a GAPDH control riboprobe. Protected RNAs were separated on a 6% polyacrylamide gel and exposed to film.

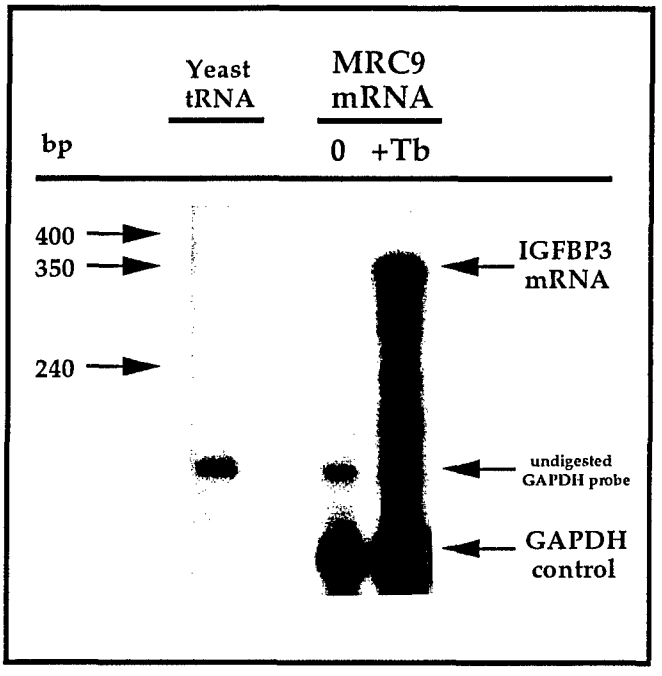


Figure 3. Induction of IGFBP3 mRNA by TGFβ in MRC9 Fibroblast cells. MRC9 cells were grown in the presence of TGFβ for 8h. Cytoplasmic RNA was isolated and hybridized with an IGFBP3 riboprobe. A control GAPDH probe was also used to verify RNA levels used in the RNase Protection.

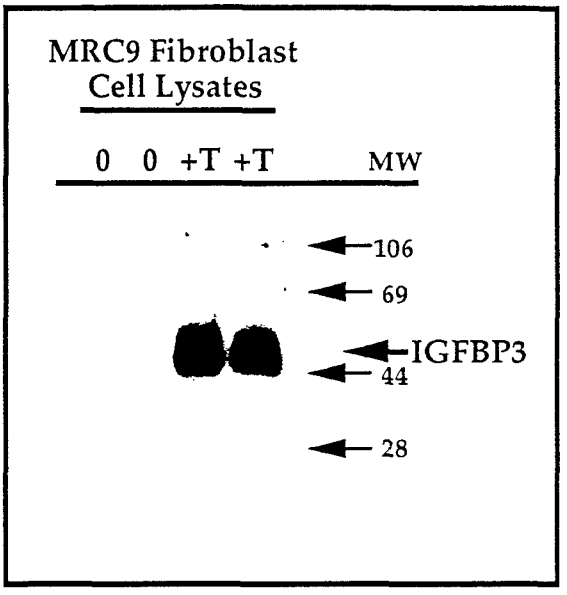


Figure 4. Induction of IGFPB3 protein by TGFβ in MRC9 fibroblast cells. Cells were incubated in serum-free media for 48h prior to TGFβ addition. The cells were then incubated with and without TGFβ for an additional48h, lysed, electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose, and assayed for IGFBP3 protein.

E. REFERENCES

1. Cullen, K.J. and Lippman, M.E. (1992) Stromal-epithelial interactions in breast cancer. Cancer Treat. Res. *61*, 413-431.

2. Roberts, A.B. and Sporn, M.B. (1990) The transforming growth factor ß, in: "Handbook of experimental pharmacology, peptide growth factors and their receptors". (M.B. Sporn and Roberts, A.B., eds.), Springer, Heidelberg.

3. Massague, J. (1990) The transforming growth factor-ß family. Annu. Rev. Cell Biol. *6*, 597-641.

4. Sporn, M.B. and Roberts, A.B. (1985) Autocrine growth factors and cancer. Nature *313*, 745-47.

5. Sun, L.-Z., Wu, G., Willson, J.K.V., Zborowska, E., Yang, J., Rajkarunanayake, I, Wang, J., Gentry, L., Wang, X.-F., Brattain, M.G. (1994) Expression of transforming growth factor ß type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J. Biol. Chem. 269, 26449-26455.

6. Wang, T., Donahoe, P.K., and Zervos, A. Specific interaction of type I receptors of the TGF-ß family with the immunophilin FKBP-12. Science, *265*: 674-676, 1994.

7. Chen, R.-H., Miettinen, P.J., Maruoka, E.M., Choy, L., and Derynck, R. A (1995) WD-domain protein that is associated with and phosphorylated by the type II TGF-ß receptor. Nature, *377*: 548-552.

8. Kawabata, M., Imamura, T., Miyazono, K., Engel, M.E., and Moses, H.L. (1995) Interaction of the transforming growth factor-ß type I receptor with farnesyl-protein transferase-a. J. Biol. Chem., *270*: 29628-29631.

9. Wang, T., Danielson, P.D., Li, B.-Y., Shah, P.C., Kim, S.D., and Donahoe, P.K. (1996) The p21RAS farnesyltransferase a subunit in TGF-ß and activin signaling. Science, *271*: 1120-1122.

10. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, E., Nishida, E., and Matsumoto, K. (1995) Identification of a member of the MAPKKK family as a potential mediator of the TGF-ß signal transduction. Science, *270*: 2008-2011.

11. Kingsley, D.M. (1994) The TGF-ß superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Devel., *8*: 133-146.

12. Seekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., and Gelbart, W.M. (1995) Genetic characterization and cloning of moters against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics, *139*: 1347-1357.

13. Savage, C., Das, P., Finelli, A.L., Townsend, S.R., Sun, C.-Y., Baird, S.E., and Padgett, R.W. (1996) Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor b pathway components. Proc. Natl. Acad. Sci. USA, *93*: 790-794.

14. Hahn, S.A., Schutte, M., Hoque, A.T.M.S., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H., and Kern, S.E. (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science, *271*: 350-353.

15. Artega, C.L., Kitten, L., Coronado, E., Jacobs, S., Kull, F.C., Allred, D.C., and Osborne, C.K. (1989) Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. J. Clin. Invest. *84*, 1418-1423.

16. Baxter, R.C. and Martin, J.L. (1989) Binding proteins for the insulin-like growth factors: structure, regulation, and function. Prog. Growth Factor Res. *1*, 49-68.

17. Oh, Y., Muller, H.L., Pham, H., and Rosenfeld, R.G. (1993) Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J. Biol. Chem. *268*, 26045-26048.

18. Conover, C.A., Clarkson, J.T., Durham, S.K.,and Bale, L.K. (1991) Cellular actions of insulin-like growth factor binding protein-3, in: "Current Directions in Insulin-like Growth Factor Research", CRC Press, pp. 255-266.

19. Ellis, M.J.C., Singer, C., Hornby, A., Rasmussen, A., and Cullen, K.J. (1994) Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer. Br. Can. Res. Treat. *31*, 249-261.

20. Gucev, Z.S., Oh, Y., Kelley, K.M.,and Rosenfeld, R.G. (1996) Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor ß2-induced growth inhibition in human breast cancer cells. Cancer Res. *56*, 1545-1550.

21. Martin, J.L., Ballesteros, M. and Baxter, R.C. (1992) Insulin-like growth factor-1 (IGF-1) and transforming growth factor-ß1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. Endocrinology *131*, 1703-1710.

22. Huynh, H., Yang, X., and Pollak, M. (1996) Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. J. Biol. Chem. *271*, 1016-1021.

23. Liu, F., Ventura, F., Doody, J., and Massague, J. (1995) Human type II receptor for bone morphogenic proteins (BMPs): Extension of the two-kinase receptor model to the BMPs. Mol. Cell Biol., *15*: 3479-3486.

24. Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Mechanism of activation of the TGF-b receptor. Nature, *370*: 341-347.

25. DeGregori, J., Kowalik, T., and Nevins, J.R. (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol. Cell Biol., *15*: 4215-4224.

26. Cohen, G.B., Ren, R., and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. Cell, *80*: 237-248.

27. Hannon, G.J. and Beach, D. (1994) p15INK4B is a potential effector of TGF-ß-induced cell cycle arrest. Nature, *371*: 257-261.

28. Datto, M.B., Li, Y., Panus, J., Howe, D.J., Xiong, Y., and Wang, X-Y. (1995) TGF-ß mediated growth inhibition is associated with induction of the cyclin-dependent kinase inhibitor, p21. Proc. Natl. Acad. Sci. USA, *92*: 5545-5549.

29. Polyak, K., Kato, J., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M., and Koff, A. (1994) p27Kip1, a cyclin-cdk inhibitor, links transforming growth factor-ß and contact inhibition to cell cycle arrest. Genes Devel., *8*: 9-22.

30. Toyoshima, H. and Hunter, T. (1994) p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. Cell *78*, 67-74.

31. Li, J.-M., Nichols, M.A., Chandrasekharan, S., Xiong, Y. and Wang, X.-F. (1995) Transforming growth factor ß activates the promoter of cyclin-dependent kinase inhibitor $p15^{INK4B}$ through an Sp1 consensus site, J. Biol. Chem. *270*, 26750-26753.

32. Rohlik, Q.T., Adams, B., Kull, F.C., Jacobs, S. (1987) An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF7 cells in tissue culture. BBRC *149*, 276-281.

33. Cubbage, M.L., Suwanichkul, A. and Powell, D.R. (1990) Insulin-like growth factor binding protein-3: Organization of the human chromosomal gene and demonstration of promoter activity. J. Biol. Chem. *265*, 12642-12649.

34. Buckbinder, B., Talbott, R., Velasco-Migel, S., Takenaka, I., Faha, B., Seizinger, B.R., and Kley, N. (1995) Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature *377*, 646-649.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 000–000, ●●●●●●●● 1996
Biochemistry

Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth

JONATHAN M. YINGLING*, PRADEEP DAS†, CATHY SAVAGE†, MING ZHANG*, RICHARD W. PADGETT†, AND XIAO-FAN WANG*‡

*Department of Pharmacology, Duke University, Durham, NC 27710; and †Waksman Institute, Department of Molecular Biology and Biochemistry and Cancer Institute of New Jersey, P.O. Box 759, Rutgers University, Piscataway, NJ 08855-0759

Communicated by Gordon G. Hammes, Duke University Medical Center, Durham, NC, May 29, 1996 (received for review April 23, 1996)

ABSTRACT **The Dwarfin protein family has been genetically implicated in transforming growth factor β (TGF-β)-like signaling pathways in *Drosophila* and *Caenorhabditis elegans*. To investigate the role of these proteins in mammalian signaling pathways, we have isolated and studied two murine dwarfins, Dwarfin-A and Dwarfin-C. Using antibodies against Dwarfin-A and Dwarfin-C, we show that these two dwarfins and an immunogenically related protein, presumably also a dwarfin, are phosphorylated in a time- and dose-dependent manner in response to TGF-β. Bone morphogenetic protein 2, a TGF-β superfamily ligand, induces phosphorylation of only the related dwarfin protein. Thus, TGF-β superfamily members may use overlapping yet distinct dwarfins to mediate their intracellular signals. Furthermore, transient overexpression of either Dwarfin-A or Dwarfin-C causes growth arrest, implicating the dwarfins in growth regulation. This work provides strong biochemical and preliminary functional evidence that Dwarfin-A and Dwarfin-C represent prototypic members of a family of mammalian proteins that may serve as mediators of signaling pathways for TGF-β superfamily members.**

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide hormone that elicits a wide range of cellular effects, including inhibition of cellular proliferation and transcriptional activation of specific target genes (1, 2). The TGF-β signal is initiated through a heteromeric transmembrane kinase complex of type I and type II receptors (3–6). A potential mechanism of activation for the heteromeric TGF-β receptor complex has been proposed (7). Within the heteromeric complex, the type II receptor phosphorylates the type I receptor, and activation of the type I receptor initiates the intracellular signaling pathway. However, the cytoplasmic signaling pathway(s) that mediate the TGF-β signal are poorly understood. Although a few receptor-interacting proteins have been identified by the yeast two-hybrid system (8–11) and one potential downstream kinase has been implicated in the TGF-β pathway (12), the precise roles for these proteins in TGF-β signaling remain to be elucidated.

Potential insight into the components of the TGF-β signaling pathways has come from the genetic isolation of a novel family of proteins in *Drosophila* and *Caenorhabditis elegans*. Decapentaplegic (dpp) is the TGF-β-like ligand in *Drosophila* (13). Genetic screens for dominant enhancers of a weak *dpp* allele led to the isolation of MAD (Mothers against dpp) (14). Loss-of-function mutations in MAD result in similar phenotypic defects as seen with mutant *dpp* alleles. thus implicating MAD in some aspect of dpp function. *C. elegans* has three MAD homologs, SMA-2, SMA-3, and SMA-4, which have been implicated in the TGF-β-like pathway in the nematode (15). These four genes define a novel family of proteins called dwarfins (15). Mutant alleles of these genes in *C. elegans* give rise to small worms and fused male tail rays. This phenotype is similar to mutant type II receptor (*daf-4*) alleles in *C. elegans* (16), thus implicating the *sma* genes in a pathway downstream of *daf-4* (15). Although the genetic evidence strongly suggests that MAD and the SMA proteins participate in TGF-β superfamily signaling pathways in *Drosophila* and *C. elegans*, the biochemical and functional nature of these proteins remains unknown.

These genetic studies prompted us to investigate the potential role of dwarfins in TGF-β signaling pathways in mammalian cells. A human dwarfin homolog, DPC4, has been identified as a candidate tumor suppressor gene in pancreatic carcinomas (17). Therefore, the dwarfins may play an important role in cellular growth control, including the ability to mediate the growth inhibitory signal initiated by TGF-β or TGF-β superfamily members. We report here the isolation and characterization of two murine dwarfins, Dwarfin-A and Dwarfin-C. Antibodies against these two proteins reveal that three endogenous dwarfins are inducibly phosphorylated in response to TGF-β, but only one of these is phosphorylated in response to bone morphogenetic protein 2 (BMP-2). This is the first indication that the signals for TGF-β superfamily members may be mediated by overlapping, but distinct, intracellular signaling pathways. Inducible phosphorylation of the dwarfins in mammalian systems provides strong support to the genetic evidence that these proteins are mediating some aspect of TGF-β superfamily signaling pathways. Furthermore, transient overexpression of either Dwarfin-A or Dwarfin-C causes a growth arrest that is consistent with their potential role in mediating TGF-β's growth inhibitory signal.

MATERIALS AND METHODS

Cell Lines. NMuMg (normal murine mammary gland epithelial) and L6 (rat skeletal muscle myoblasts) cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Cloning of Dwarfin cDNAs. A 180-nt fragment of Dwf-A was generated by degenerate PCR (15) and used to screen a 12.5-day mouse embryo library to obtain a partial Dwf-A cDNA of 650 bp. The 650-bp *Eco*RI/*Xho*I clone was radiolabeled with [^{32}P]dCTP using the Prime-It II kit from Stratagene

Abbreviations: TGF-β, transforming growth factor β; BMP-2, bone morphogenetic protein 2; dpp, decapentaplegic; NMuMg, normal murine mammary gland epithelial cells; BrdU, ●●●●: DH1 and DH2, dwarfin homology domains 1 and 2;

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U58992 (Dwarfin-A) and U58993 (Dwarfin-C)].

‡To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

and used to screen a λgt10 8.5-day mouse embryonic library at low stringency. Briefly, hybridization was at 42°C in 45% formamide, 5× standard saline phosphate/EDTA (SSPE; 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA for 16–20 hr. The filters were washed two times at RT in 2× standard saline citrate (SSC)/0.1% SDS, one time at RT in 0.5× SSC/0.1% SDS, and one time in 0.5× SSC/0.1% SDS at 55°C and were exposed overnight. Positive plaques were purified through quaternary screens before the insert was PCR amplified using λgt10-specific PCR primers (5′ primer, AGCAAGTTCAGCCTGGTTAAG; 3′ primer, 5′-TTATGAGTATTTCTTCCAGGG). PCR products were subcloned into pGEM-T (Promega) and partially sequenced with T7 and SP6 primers. This approach isolated a Dwf-A cDNA of 1639 nucleotides and a Dwf-C cDNA of 2185 nucleotides. Both contain an open reading frame of 465 amino acids with a predicted molecular mass of 52 kDa. Subcloning and deletional analysis combined with automated sequencing yielded the nucleotide sequences of Dwf-A and Dwf-C.

Mammalian expression constructs were constructed using *Bam*HI fragments containing full-length cDNAs for Dwf-A and Dwf-C generated by PCR using the following primer sets: Dwf-A 5′ primer, CGCGGATCCGCGATGAATGTGACCAGCTTG; Dwf-A 3′ primer, CGCGGATCCGCGCAGAGTTACCAGGTTTGGC; Dwf-C 5′ primer, CGGGATCCCGGAATTCCATGACGTCAATGGCCAGC; and Dwf-C 3′ primer, CGCGGATCCGCGGTAAGGCAAAGAAATTCC. The resulting PCR products were subcloned into pGEM-T and subsequently into pCMV5 and confirmed by sequencing with a cytomegalovirus promoter-specific sequencing primer: 5′-GCGGTAGGCGTGTACGG-3′.

Northern Analysis. A multiple-tissue Northern blot (CLONTECH) containing 2 μg of poly(A)$^+$ RNA was sequentially probed with the 1.6-kb full-length Dwf-A cDNA followed by the 2.1-kb full-length Dwf-C cDNA. The probes were labeled with [α-^{32}P]dCTP by random priming (Stratagene) and hybridized for 16–20 hr at 42°C in 50% formamide. 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. The filters were washed two times at RT in 2× SSC/0.1% SDS and two times in 0.2× SSC/0.1% SDS at 60°C. Autoradiography was at −80°C for 18–24 hr with intensifying screens.

Antibody Production. Bacterial expression constructs for Dwf-A and Dwf-C were constructed using the *Bam*HI fragments from pGEM-T Dwf-A or Dwf-C, respectively, to clone in-frame into pGex 2T, generating glutathione *S*-transferase fusion proteins. The resulting ≈85-kDa fusion proteins were used as antigens for rabbit polyclonal antibody production. Preimmune sera were obtained from each animal before the primary injection of antigen. Both the Dwf-A and Dwf-C antibodies specifically recognize 56-kDa and 52-kDa proteins on Western blots of NMuMg or L6 lysates (data not shown). In addition, transfection of Dwf-A or Dwf-C cDNA into COS cells generates a tight doublet around 52 kDa which cannot account for the two bands seen by Western blotting of endogenous proteins (data not shown). Thus, the 56-kDa protein is most likely immunogenically related to the dwarfins and represents an additional dwarfin family member.

***In Vivo* Phosphorylation of Endogenous Dwarfins.** NMuMg or L6 cells were plated at 1.5 × 10^6 cells per 100-mm dish and allowed to attach overnight. The cells were rinsed once with phosphate-free media (ICN) and then starved in phosphate-free media containing 0.5% dialyzed FBS (GIBCO/BRL) for 1 hr and then labeled in 0.5% dFBS with 0.5 mCi/ml (1 Ci = 37 GBq) [^{32}P]orthophosphate for 4 hr. TGF-β1 or BMP-2 was added at various concentrations for the indicated length of time at the end of the labeling period. Cells were rinsed twice with ice-cold PBS and lysed in 50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 0.5 mM Na_2MoO_4, 1 mM DTT, 1× protease inhibitors (5 μg/ml antipain, aprotinin, leupeptin, and trypsin inhibitor; 0.5 μg/ml pepstatin), and 1 mM phenylmethylsulfonyl fluoride on ice for 15 min. Lysates were microcentrifuged at 14,000 rpm at 4°C for 15 min. The resulting cell lysates were precleared with Protein-A Sepharose for 30 min at 4°C. Lysates were then divided for immunoprecipitation with Dwf-A or Dwf-C antibodies. Preimmune or immune sera (20 λ of unpurified sera) was added with Protein-A Sepharose for 4 hr at 4°C. The immunoprecipitates were washed three times with lysis buffer before separation in SDS/8% polyacrylamide followed by autoradiography at room temperature.

Transient Growth Arrest Assay. The following procedure was adapted from DeGregori and coworkers (18). L6 cells were plated in six-well trays on Poly-L-Lysine-coated coverslips at 10^5 cells per well and allowed to attach overnight. CellFectin (GIBCO/BRL) was used to transfect the cells with 2 μg of pCMV LacZ and 8 μg of vector alone, Dwf-A, or Dwf-C for 10 hr in DMEM. The cells were incubated in 10% FBS for 36 hr before immunohistochemical staining. Media containing 20 μM BrdU was added for the last 20 hr to metabolically label dividing cells. The cells were fixed in 4% paraformaldehyde in PBS, permeabilized with MeOH/acetone, and stained with a 1:500 dilution of rabbit anti-β-galactosidase antibody (5 Prime–3 Prime, Inc.) in 1% BSA/PBS for 60 min, followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim) at 1:500 in 1% BSA/PBS to stain transfected cells. After a second fixation and 2 M HCl permeabilization for 45 min, the cells were stained with a mouse anti-BrdU antibody (Zymed) at 1:3 in 1% BSA/PBS followed by a goat anti-mouse Rhodamine-conjugated secondary antibody (Pierce) at 1:100 in 1% BSA/PBS. Finally, 4 μg/ml Hoeschst 33342 in PBS was used to counter-stain the DNA. The coverslips were mounted in DABCO and analyzed by fluorescence microscopy.

RESULTS

Cloning of Dwarfin-A and Dwarfin-C. Full-length cDNAs for Dwf-A and Dwf-C were isolated by low stringency hybridization of a 8.5-day mouse embryonic library as described in *Materials and Methods*. The amino acid sequences of Dwf-A and Dwf-C are 95% homologous and 90% identical. Both contain the characteristic dwarfin homology domain 1 (DH1) and dwarfin homology domain 2 (DH2) motifs separated by a proline-rich linker region (Fig. 1*A*; ref. 15). Dwf-A and Dwf-C are about 80% homologous to the *Drosophila* dwarfin, MAD (Fig. 1*A*; ref. 14), and the *C. elegans* dwarfin, SMA-2 (Fig. 1*A*; ref. 15). Dwf-A and Dwf-C are distantly related to the only other known mammalian dwarfin, DPC4 (60% homologous, 40% identity; ref. 17), and thus likely represent a distinct family of mammalian dwarfins (Fig. 1*B*). Northern analysis showed that both genes are ubiquitously expressed (Fig. 1*C*). The mRNA message of Dwf-A is ≈3.5 kb, whereas Dwf-C has two messages of ≈8 and ≈3 kb. F1

TGF-β Induces Phosphorylation of Endogenous Dwarfins. We chose two cell lines, NMuMg (19) and L6 (which are potently growth inhibited by TGF-β), as model systems to study endogenous dwarfin phosphorylation. Antibodies against Dwf-A or Dwf-C were used to determine if the levels or phosphorylation state of endogenous dwarfins were modulated by TGF-β. The expression levels of dwarfins were unchanged at any time within 24 hr after TGF-β treatment as determined by Western blot analysis (data not shown). However, TGF-β induced rapid, but transient, phosphorylation of Dwf-A, Dwf-C, and a 56-kDa immunogenically related protein (Fig. 2*A*). F2

Phosphorylation of Dwf-A and Dwf-C was induced within 15 min and peaked at 2.4-fold and 4-fold by 60 min, respectively (Fig. 2*B*). The difference in the extent of phosphorylation of

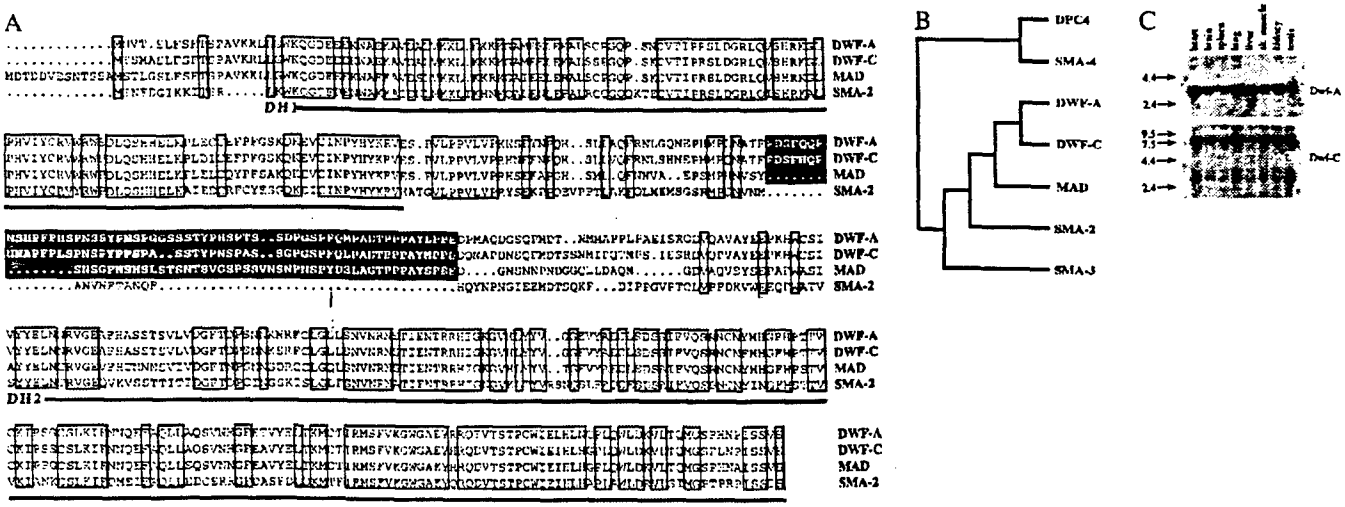


FIG. 1. Predicted amino acid sequence and expression pattern of Dwf-A and Dwf-C. (*A*) Alignment of Dwf-A and Dwf-C with *Drosophila* MAD and *C. elegans* SMA-2. The GenBank accession numbers for Dwf-A and Dwf-C are U58992 and U58993, respectively. The two highly conserved domains, DH1 and DH2, are underlined. The serine-threonine-rich insert in the mammalian dwarfins is highlighted. (*B*) Dendrogram analysis of the dwarfin protein family. (*C*) Northern analysis of Dwf-A and Dwf-C. A rat multiple tissue Northern blot was sequentially probed with the 1.6-kb full-length Dwf-A cDNA followed by the 2.1-kb full-length Dwf-C cDNA.

Dwf-A and Dwf-C is due to the lack of basal phosphorylation of Dwf-C in the absence of TGF-β treatment (Fig. 2*A*). Phosphorylation of the 56-kDa protein in both αDwf-A and αDwf-C immunoprecipitates was also induced within 15 min and peaked at 3.2-fold by 60 min (Fig. 2*B*). By 4 hr the phosphorylation state of all three proteins had returned to nearly basal levels. The induced phosphorylation of Dwf-A, Dwf-C, and the 56-kDa protein was primarily on serine residues (≈90%) with minor threonine phosphorylation (≈10%) as determined by phosphoamino acid analysis (data not shown).

Immunoprecipitation of Dwf-A and Dwf-C from L6 cells gave nearly identical results as those from NMuMg cells (Fig. 2 *C* and *D*). Dwf-A and the 56-kDa protein were phosphorylated to a similar extent and with similar kinetics in both cell lines. Dwf-C was phosphorylated with the same kinetics as in NMuMg cells, but with a reduced extent due to increased basal phophorylation in the absence of TGF-β in L6 cells (Fig. 2*C*).

To ensure that TGF-β was capable of inducing phosphorylation of the dwarfins at more physiological concentrations, we determined the dose dependence of dwarfin phosphorylation in NMuMg cells. Dwf-A, Dwf-C, and the 56-kDa protein were all inducibly phosphorylated by 10 pM TGF-β (Fig. 3), a concentration capable of eliciting the various biological effects of TGF-β. F3

BMP-2 Induces Phosphorylation of Endogenous Dwarfins. TGF-β-like ligands from *Drosophila* and *C. elegans* are most closely related to mammalian BMPs (16, 20). BMPs have been shown to elicit multiple effects, including inhibition of cellular proliferation, on many different cell types (21). Like TGF-β, BMP-2 has been shown to initiate its signaling cascade by binding to a heteromeric complex of transmembrane serine-threonine kinase receptors at the cell surface (22). However, the similarity between the cytoplasmic pathways that lead to the biological effects of TGF-β and BMP-2 is unknown. The NMuMg cell line is potently inhibited by BMP-2, affording us

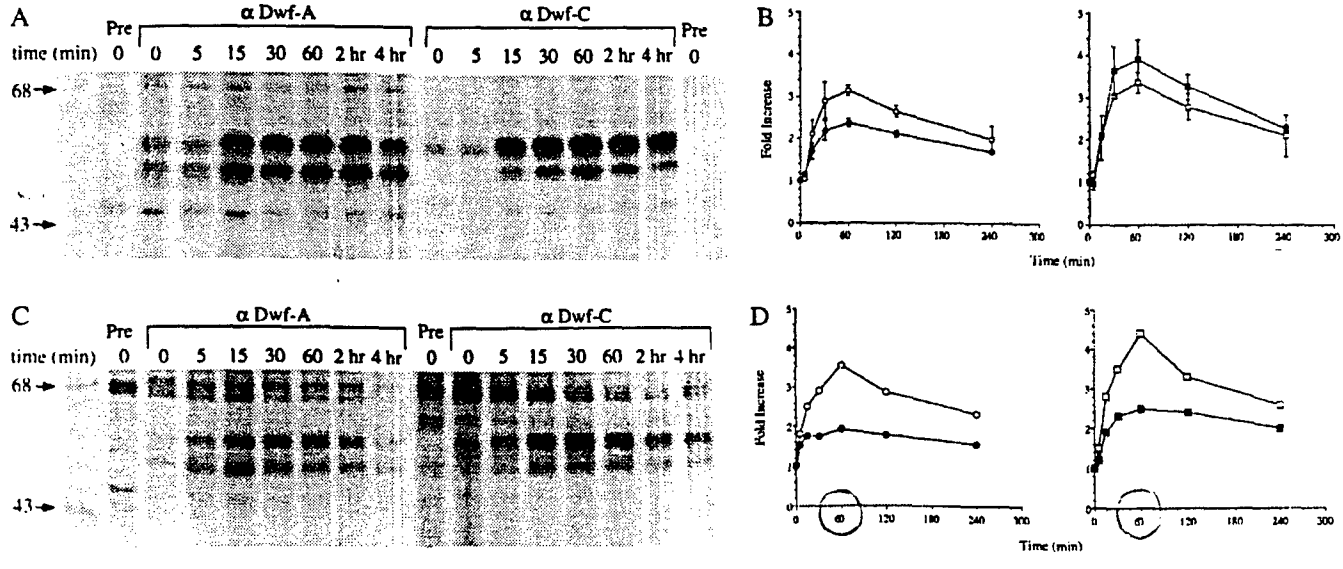


FIG. 2. TGF-β-induced phosphorylation of endogenous dwarfins. Immunoprecipitation with Dwf-A (*Left*) or Dwf-C (*Right*) antibodies was performed on NMuMg (*A*) or L6 (*B*) cells treated for the indicated length of time with 500 pM (7.5 ng/ml) TGF-β1. Quantitation of TGF-β induced dwarfin phosphorylation in NMuMg (*C*) or L6 (*D*) cells. The 52-kDa Dwf-A (●) and Dwf-C (■) bands were quantitated using a phosphoimager. The 56-kDa protein in αDwf-A (○) or αDwf-C (□) immunoprecipitates was also quantitated. The level of phosphorylation was standardized using the 68-kDa background band and two additional background bands.

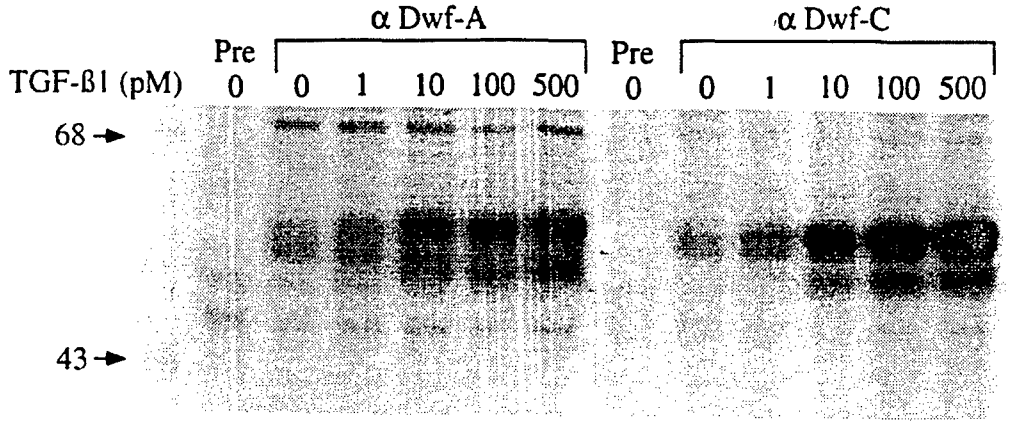


FIG. 3. TGF-β phosphorylation of endogenous dwarfins is dose-dependent. Dwf-A and Dwf-C immunoprecipitation from NMuMg cells was performed as in Fig. 2 except various doses of TGF-β1 were added for 1 hr.

the opportunity to determine if dwarfin phosphorylation is induced by BMP-2. As shown in Fig. 4 *A* and *B*, phosphorylation of the 56-kDa protein is induced by BMP-2 within 15 min and peaks at 2.5-fold in 60 min. Interestingly, Dwf-A and Dwf-C, which are inducibly phosphorylated in response to TGF-β, are not phosphorylated in BMP-2-treated NMuMg cells (Fig. 4*A*).

Dwf-A and Dwf-C Are Implicated in Cell Growth Regulation. Attempts to establish stable cell lines constitutively overexpressing either Dwf-A in L6 cells or Dwf-C in NMuMg cells were unsuccessful. This result was not unexpected because the dwarfins are suspected to have growth-suppressive effects based on the tumor suppressor activity of DPC4 (17). Consequently, we used a modified transient growth assay (18) to assess the ability of Dwf-A or Dwf-C to cause a growth arrest when transiently transfected into L6 cells. Constitutive overexpression of Dwf-A or Dwf-C caused 30–40% growth inhibition compared with 10% for control vector transfectants (Fig. 5). Therefore, Dwf-A and Dwf-C, like DPC4, exhibit growth-inhibitory properties, implicating these dwarfin proteins in cell growth regulation.

DISCUSSION

Identification of downstream effectors for TGF-β or TGF-β superfamily members has proven elusive. Mutagenesis studies in mammalian cells have yielded only receptor mutants (23, 24), which suggests the existence of redundant pathways downstream of the receptors. Fortunately, TGF-β-like pathways exist in genetically tractable organisms to allow the use of genetics to identify components of these signaling pathways. Many of these components (e.g., receptors, accessory molecules, and ligands) have been shown to have homologous counterparts in vertebrate systems. Consequently, we have studied two mammalian homologs of MAD and SMA-2 as potential downstream effectors of the TGF-β signaling pathway. We provide biochemical evidence that the dwarfin family of proteins is involved in TGF-β and BMP-2 signaling pathways in mammalian systems. Furthermore, results from a modified transient growth assay and preliminary studies with potentially dominant negative forms of Dwarfin-A (unpublished data) strongly implicate a role for the dwarfins as mediators of the TGF-β growth-regulatory signal.

Although the dwarfins do not contain any known catalytic motifs, their DH1 and DH2 domains are reminiscent of Src homology 2 and 3 domains, which in a variety of signaling pathways modulate protein–protein interactions based on tyrosine phosphorylation and proline-rich sequences, respectively (25). TGF-β and BMP-2-induced phosphorylation of the dwarfins may regulate protein–protein interactions in an analogous fashion for TGF-β superfamily signaling cascades. Recently, 14-3-3 proteins have been shown to be specific phosphoserine-binding proteins that are critical for the activation of signaling proteins (26). This suggests a novel role for serine–threonine phosphorylation in the assembly of protein–protein complexes required to transduce certain intracellular signals. Serine–threonine phosphorylation of the dwarfins may regulate their ability to serve as adaptor molecules for other effectors in the TGF-β pathway or regulate their ability to specifically bind other intracellular proteins. These protein–protein interactions may result in altered subcellular distribution of the dwarfins. Preliminary immunofluorescence studies in NMuMg cells indicate that the dwarfins are predominantly localized in the cytoplasm (unpublished data). Although TGF-β or BMP-2 treatment does not appear to cause a significant change in the subcellular distribution of the dwarfins, it is possible that a minor proportion of dwarfins that become phosphorylated accumulate at the membrane or trans-

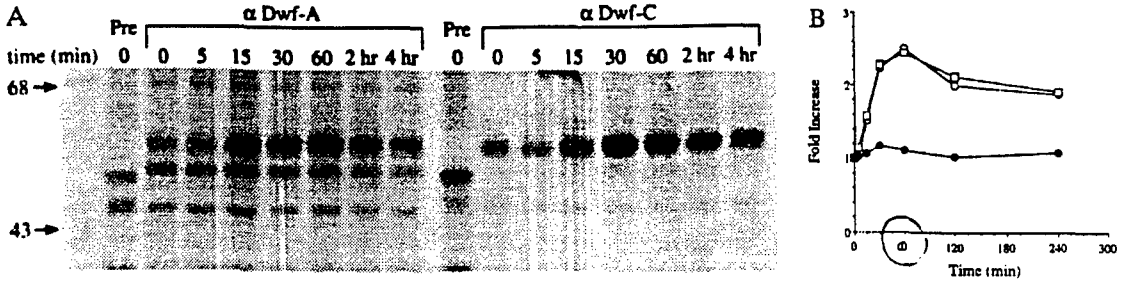


FIG. 4. BMP-2 induced phosphorylation of endogenous Dwarfins. (*A*) Immunoprecipitation with Dwf-A (*Left*) or Dwf-C (*Right*) antibodies was performed on NMuMg cells treated for the indicated length of time with 100 ng/ml BMP-2. (*B*) Quantitation of BMP-2 induced dwarfin phosphorylation in NMuMg cells. The 52-kDa Dwf-A band (●) and the 56-kDa protein in αDwf-A (○) or αDwf-C (□) immunoprecipitates were quantitated. The 52-kDa Dwf-C band that is not basally phosphorylated in NMuMg cells is undetectable after BMP-2 treatment. The level of phosphorylation was standardized using the 68-kDa background band and two additional background bands.

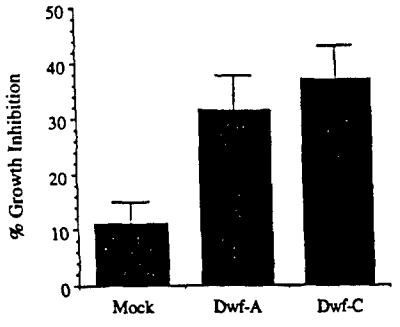


FIG. 5. Dwf-A and Dwf-C cause a growth arrest in L6 cells. L6 cells were transfected and immunohistochemically stained as described in *Materials and Methods*. All the transfected cells (fluorescein isothiocyanate stained) on each coverslip were scored for BrdU incorporation (Rhodamine stained). All (100%) of the nontransfected cells incorporate BrdU during the labeling period; therefore, the percentage of transfected cells that are BrdU-negative represents the percent growth inhibition caused by transfection of the cDNA. Data shown are the mean ± SD of at least three experiments.

locate to the nucleus to fulfill their biological function. Indeed, a human homolog of Dwf-A, MADR1, has recently been shown to be inducibly phosphorylated and to translocate to the nucleus after BMP-2 treatment (27). Intriguingly, phosphorylation of MADR1 appears to be BMP-2-specific in their system, since neither TGF-β nor activin induces MADR1 phosphorylation. This apparent discrepancy may be due to differences in experimental systems, overexpression of epitope-tagged MADR1 instead of endogenous dwarfins, or may represent cell-type specific differences in the pathways used by TGF-β superfamily ligands. Clarification of this issue requires further study.

Our initial attempt to study TGF-β's ability to modulate the phosphorylation state of Dwf-A and Dwf-C involved transfection of hemagglutinin epitope-tagged cDNAs into COS or mink lung epithelial cells. Although both proteins are phosphorylated in these systems, the high level of constitutive phosphorylation precluded detection of TGF-β induced changes in Dwf-A or Dwf-C phosphorylation (unpublished data). Interestingly, the *C. elegans* dwarfins, SMA-2 and SMA-3, are not phosphorylated when overexpressed in COS cells. Therefore, the observed phosphorylation is likely a result of phosphorylation by an associated kinase that is unable to recognize SMA-2 or SMA-3 as substrates. The unique serine-threonine-rich insert in the linker region of Dwf-A and Dwf-C (Fig. 1*A*) may play a role in either kinase recognition or as targets of phosphorylation by the associated kinase. Preliminary results indicate that neither Dwf-A nor Dwf-C are substrates of the type I or type II TGF-β receptor kinases (unpublished data), implicating an as yet unidentified kinase in phosphorylation of the dwarfins.

The differential phosphorylation of endogenous dwarfins in response to TGF-β and BMP-2 suggests that the specific dwarfins used by TGF-β superfamily members may vary. In support of this notion, two related *Xenopus* dwarfins (Xmad1 and Xmad2) have been shown to mediate either BMP-2/BMP-4 signals or Vg1/activin/nodal signals, respectively (28). Thus, Dwf-A and Dwf-C may represent TGF-β-specific dwarfins, whereas the 56-kDa dwarfin is shared by TGF-β and BMP-2. The differential response of these proteins to TGF-β and BMP-2 will facilitate elucidation of the differences in the intracellular pathways used by these two members of the TGF-β superfamily.

We wish to thank S.-J. Lee for the 12.5-day mouse embryonic library and B. Hogan for the 8.5-day mouse embryonic library; Genetics Institute for recombinant human BMP-2; Amgen for recombinant human TGF-β1; Y. Xiong for automated sequencing assistance; J. Heitman and M. Caron for critical review of the manuscript; C. Bassing, D. Cortez, M. Datto, P. Hu, G. Reuther, and H. Symonds for helpful discussion; and Y. Yu and H. Wang for technical help. J.M.Y. thanks M. L. Yingling for her constant support and encouragement. This work was supported by U.S. Army Breast Cancer Research Program Grant DAMD17-945-4065 (X.-F.W.), National Institutes of Health Grant GM47395 (R.W.P.); and Council for Tobacco Research Grant 3639 (R.W.P.). J.M.Y. was supported by a U.S. Army Breast Cancer Research Program Predoctoral Fellowship (DAMD17-94-J-4190). P.D. is a Busch Predoctoral Fellow, and C.S. is a New Jersey Cancer Commission Postdoctoral Fellow; X.-F.W. is a Leukemia Society Scholar.

1. Massague, J. (1990) *Annu. Rev. Cell Biol.* **6**, 597–641.
2. Roberts, A. B. & Sporn, M. B. (1990) in *Handbook of Experimental Pharmacology, Peptide Growth Factors and their Receptors*, eds. Sporn, M. B. & Roberts, A. B. (Springer, Heidelberg), pp. 419–472.
3. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. & Lodish, H. F. (1992) *Cell* **68**, 775–785.
4. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F. & Massague, J. (1992) *Cell* **71**, 1003–1014.
5. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H. & Miyazono, K. (1993) *Cell* **75**, 681–692.
6. Bassing, C. H., Yingling, J. M., Howe, D. J., Wang, T., He, W. W., Gustafson, M. L., Shah, P., Donahoe, P. K. & Wang, X.-F. (1994) *Science* **263**, 87–89.
7. Wrana, J. L., Attisano, L., Weiser, R., Ventura, F. & Massague, J. (1994) *Nature (London)* **370**, 341–347.
8. Wang, T. W., Donahoe, P. K. & Zervos, A. S. (1994) *Science* **265**, 674–676.
9. Chen, R. H., Miettinen, P. J., Maruoka, E. M., Choy, L. & Derynck, R. (1995) *Nature (London)* **377**, 548–552.
10. Kawabata, M., Imamura, T., Miyazono, K., Engel, M. E. & Moses, H. L. (1995) *J. Biol. Chem.* **270**, 29628–29631.
11. Wang, T. W., Danielson, P. D., Li, B.-y., Shah, P. C., Kim, S. D. & Donahoe, P. K. (1996) *Science* **271**, 1120–1122.
12. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. & Matsumoto, K. (1996) *Science* **270**, 2008–2011.
13. Padgett, R. W., St. Johnston, R. D. & Gelbart, W. M. (1987) *Nature (London)* **325**, 81–84.
14. Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. & Gelbart, W. M. (1995) *Genetics* **139**, 1347–1358.
15. Savage, C., Das, P., Finelli, A. L., Townsend, S. R., Sun, C.-Y., Baird, S. E. & Padgett, R. W. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 790–794.
16. Estevez, M., Attisano, L., Wrana, J. L., Albert, P. S., Massague, J. & Riddle, D. L. (1993) *Nature (London)* **364**, 644–649.
17. Hahn, S. A., Schutte, M., Shamsul Hoque, A. T. M., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H. & Kern, S. E. (1996) *Science* **271**, 350–353.
18. DeGregori, J., Kowalik, T. & Nevins, J. R. (1995) *Mol. Cell Biol.* **15**, 4215–4224.
19. Miettinen, P. J., Ebner, R., Lopez, A. R. & Derynck, R. (1994) *J. Cell Biol.* **127**, 2021–2036.
20. Maliakal, J. C., Asahina, I., Hauschka, P. V. & Sampath, T. K. (1994) *Growth Factors* **11**, 227–234.
21. Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C.-H. & Miyazono, K. (1995) *J. Cell Biol.* **130**, 217–226.
22. Liu, F., Ventura, F., Doody, J. & Massague, J. (1995) *Mol. Cell. Biol.* **15**, 3479–3486.
23. Boyd, F. & Massague, J. (1989) *J. Biol. Chem.* **264**, 2272–2278.
24. Laiho, M., Weis, F. M. B. & Massague, J. (1990) *J. Biol. Chem.* **265**, 18518–18524.
25. Cohen, G. B., Ren, R. & Baltimore, D. (1995) *Cell* **80**, 237–248.
26. Muslin, A. J., Tanner, J. W., Allen, P. M. & Shaw, A. S. (1996) *Cell* **84**, 889–897.
27. Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L. & Wrana, J. L. (1996) *Cell* **85**, 489–500.
28. Graff, J. M., Bansal, A. & Melton, D. A. (1996) *Cell* **85**, 479–487.